



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A23K 1/16, 1/18	A1	(11) International Publication Number: WO 00/62625 (43) International Publication Date: 26 October 2000 (26.10.00)
(21) International Application Number: PCT/NO00/00129 (22) International Filing Date: 17 April 2000 (17.04.00) (30) Priority Data: 19991857 19 April 1999 (19.04.99) NO (71) Applicant (for all designated States except US): NORSK HYDRO ASA [NO/NO]; N-0240 Oslo (NO). (72) Inventors; and (75) Inventors/Applicants (for US only): BREIVIK, Harald [NO/NO]; Uranusveien 22, N-3942 Skjelsvik (NO). SANNA, Lola, Irene [NO/NO]; Borgåsveien 10, N-3910 Porsgrunn (NO). AANESEN, Berit, Annie [NO/NO]; Nedre Kongerød 46, N-3737 Skien (NO). (74) Agent: SANDBU, Elisabeth, Dahl; Norsk Hydro ASA, N-0240 Oslo (NO).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: PIGMENT (57) Abstract <p>This invention relates to a new pigment in feed for salmonides, a new feed comprising this pigment and use of this pigment. The pigment comprises a diester of astaxanthin prepared with an omega-3 fatty acid and/or a short chain carboxylic acid. By this invention a pigment for feed to salmonides that is more stable and biologically more effective than free astaxanthin and commercially available astaxanthin products, is provided.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

"Pigment"

This invention relates to a new pigment in feed for salmonides, a new feed comprising this pigment and use of the pigment.

5

In feed for farmed salmon and trout pigment has to be added to obtain the desired colour of the fish flesh. The pigment mostly used is astaxanthin which corresponds to the pigment which is available in feed for wild salmonides. Also other pigments like for instance cantaxanthin, might be used. Such pigments are very unstable
10 with regard to exposure to air and temperature as well as light. The pigments are therefore to a great extent degraded during feed processing and storage. These pigments are all carotenoids. This feed is mostly prepared from raw material not containing significant amounts of astaxanthin (i.e. white fish). Farmed salmon and trout is fed industrially manufactured feed where pigment is added.

15

Commercially available astaxanthin products are furthermore very expensive and their biological retention is very low. Astaxanthin is as mentioned above a rather unstable compound, which of course is a drawback. The low stability of astaxanthin is due to oxidation. Commercial pigment products are formulated in order to avoid
20 or reduce oxidation. One typical formulation for astaxanthin is with gelatine and starch. The formulations used are often, however, not optimal with respect to biological availability of the pigment, and a new way of solving the stability problem, combining a high degree of stability with improved biological availability would be of great economical benefit to the aquaculture industry. A more stable pigment is thus
25 highly desired as this would give possibilities for making a formulation more optimal with regard to biological availability and consequently possibilities for considerably economic saving.

Thus it is a desire in the aquaculture industry to find more stable and biologically
30 effective pigments useful in production of feed for salmonides.

The different salmonid species differ in their ability to utilise dietary carotenoid. Rainbow trout (*Oncorhynchus mykiss*) has been found to utilize the pigment in the feed more effectively than Atlantic salmon (*Salmo salar*) and sea trout (*S. trutta*).

Rainbow trout can also accumulate higher amounts of carotenoids in the flesh than Atlantic salmon and sea trout, but less than sockeye salmon (*Oncorhynchus nerka*) (Storebakken, T. and Ho, N.K., Aquaculture, vol. 100, (1992), p. 209).

- 5 In salmon, dietary astaxanthin and canthaxanthin are deposited more efficiently in flesh than in skin, which is in contrast to the rainbow trout (Schiedt, K. et al., Pure & Appl. Chem. 57 (1985) 685-692).

- Synthetically produced astaxanthin is normally present in unesterified form (i.e. diol). This is also the form assumed that the pigment is converted to in the intestine before it is absorbed by the fish (O.J. Torrissen et al., Reviews in Aquatic Sciences, vol. 1, (1989) pp. 209-225). In nature astaxanthin is often present as diester.

- Simpson, K.L. and Kamata, T., Proc. World Symp. on Finfish Nutr. and Fishfeed Technology, Hamburg 20.-23. June, 1978. Vol II. Berlin 1979, pp. 415-424, reported a study for pigmentation of rainbow trout comparing astaxanthin, astaxanthin ester and astacene. Astaxanthin, astaxanthin ester and astacene were extracted from shrimp coagulum. The pigments were dissolved in herring oil and added to the trout diet. When analysing the fish, no distinction was made between flesh and skin. The fish fed the diet consisting of astaxanthin ester contained much higher levels of total astaxanthin than others. This indicated that astaxanthin ester was more effective for the pigmentation of rainbow trout. However, on the same symposium Torrissen, O. and Brækkan, O.R. (Proc. World Symp. on Finfish Nutr. and Fishfeed Technology, Hamburg 20.-23. June, 1978. Vol II. Berlin 1979, pp. 377-382) also demonstrated that astaxanthin was incorporated into the flesh of rainbow trout. These authors found that astaxanthin was more effectively incorporated in flesh than diesters and monoesters purified from the copepod, *Calanus finmarchicus*.

- 30 According to O.J. Torrissen et al., Reviews in Aquatic Sciences, vol. 1, (1989) pp. 209-225 (i.e.: Foss, P. et al., Aquaculture, vol. 65, (1987), p.293 and Storebakken, T. et al., Aquaculture, vol. 65, (1987), p. 279) synthetic astaxanthin diester (i.e. astaxanthin dipalmitate) seems to be absorbed less easy than free astaxanthin both in rainbow trout, sea trout and Atlantic salmon.

In crustaceans, a relatively large part of the astaxanthin is present in ester form. However, the pigment is more easily absorbed than what should be expected from the level of free astaxanthin. This is in O.J. Torrissen et al., Reviews in Aquatic Sciences, vol. 1, (1989) pp. 209-225 tentatively explained from other not identified
5 compounds in crustacea which might contribute to enhanced absorption.

To summarise: as astaxanthin absorption in the intestine of the fish is assumed to involve free astaxanthin (i.e. diol) it is so far mainly considered that feeding with esters will give less biological absorption than feeding with free astaxanthin. This is
10 supported by experiments with astaxanthin dipalmitate.

It is known that astaxanthin present as diester is more stable than free astaxanthin (Omara-Alwala, T.R. et al., J. Agric. Food Chem., vol. 33 (1985), p. 260 and Arai, S. et al., Aquaculture, vol. 66 (1987), p. 255.)

15

In the literature, dipalmitate is the predominant diester studied, and it is reported to give less pigmentation than the diol (Torrissen, O. and Brækkan, O.R.; Proc. World Symp. on Finfish Nutr. and Fishfeed Technology, Hamburg 20.-23. June, 1978. Vol II. Berlin 1979, pp. 377-382, Storebakken, T. et al., Aquaculture, vol. 65
20 (1987), p. 279, Foss, P. et al., Aquaculture vol. 65 (1987), p. 293, Torrissen, O.J et al., CRC Crit. Rev. Aqua. Sci. vol. 1 (1989), p. 209.). This is explained by a low degree of hydrolysis of the diester.

We have shown that by using a commercial lipase (*Candida rugosa*), after 42
25 hours the dipalmitate hydrolysed to 40% free astaxanthin. We synthesised several other diesters in order to study whether these hydrolysed faster than the dipalmitate. Example 1 shows that under the same experimental conditions the diester with elaidic acid (trans-C18:1) hydrolysed to a higher degree (73%) while a short-chain carboxylic acid (C10:0) hydrolysed somewhat slower, and a diester
30 prepared with a concentrate of omega-3 fatty acids comprising approx. 50% EPA (all cis C20:5 n3) and approx. 35% DHA (all cis C22:6 n3) (in total more than 90% omega-3 fatty acids) (EPA+DHA) hydrolysed to free astaxanthin at less than half the rate of the dipalmitate.

To verify the hydrolysis data for these astaxanthin diesters obtained by using the commercial lipase, another similar experiment was performed by using enzyme isolated from the intestine of Atlantic salmon. This experiment very surprisingly gave opposite data than the data obtained in the experiment where the commercial lipase was used; i.e. the EPA+DHA diester was hydrolysed quickest and the dielaidate and the dipalmitate were hydrolysed slowest (see Example 2). Thus the inventors most surprisingly have found that if astaxanthin esterified with a concentrate of omega-3 polyunsaturated fatty acids is hydrolysed by enzyme from the intestine of salmon, a surprisingly fast hydrolysis to free astaxanthin is obtained compared to dipalmitate. Surprisingly, also diester with the short chain carboxylic acid (C10:0) was hydrolysed much faster than the dipalmitate, even though the rate of hydrolysis was significantly slower than hydrolysis of the EPA+DHA diester.

Based on these surprising data, the present inventors have found a astaxanthin EPA+DHA diester which most likely hydrolyses quickly to free astaxanthin when fed to the salmon and thus is effective for pigmentation of salmon. In Example 3 it is shown by feeding experiments that this statement is correct. In a similar way, the inventors have found that an astaxanthin diester with short chain carboxylic acids will be suitable as a pigment with good stability and a high potential for pigmentation of salmonides.

Furthermore, it was surprisingly found by the inventors that the EPA+DHA diester comprised acceptable stability properties for use in industrially manufactured feed without formulation with gelatine or starch (see Example 3). This was not expected, as omega-3 polyunsaturated fatty acids are unstable compounds.

In Example 3 pigmentation of salmon with the EPA + DHA diester was compared to pigmentation with a commercial free astaxanthin product (Carophyll Pink, Roche). Most surprisingly, it was found that the bioavailability as measured by astaxanthin uptake in salmon fillet was 41% higher in the fish fed the EPA + DHA diester compared with the fish fed the commercial pigment. Thus, feeding with this astaxanthin diester surprisingly gives enhanced biological absorption compared to free astaxanthin.

It is a main object of the invention to provide a pigment for feed to salmonides that is more stable and biologically effective than free astaxanthin and commercial pigments for salmonides.

5

Another object of this invention is to provide a pigment which can be added to the feed in less amounts than previously known pigments and still give a satisfactory pigmentation of the flesh.

10 This and other objects are achieved by the attached claims.

The invention is further explained by examples.

15 Example 1

Diesters of astaxanthin were prepared with the following carboxylic acids: elaidic acid (trans-C18:1), palmitic acid (C16:0), decanoic acid (C10:0) and a concentrate

20 of omega-3 fatty acids comprising approximately 50% eicosapentaenoic acid (EPA) (all cis C20:5 n3) and approximately 35% docosahexaenoic acid (DHA) (all cis C22:6 n3). The different diesters were firstly hydrolysed by a commercial lipase, *Candida rugosa* (Lipase AY, 30). This reaction gave the following results after 42 hours at room temperature:

25	Diester	Free astaxanthin (%)
	18:1	73
	16:0	40
	10:0	30
	EPA+DHA	18

30

These results show that the diester with polyunsaturated fatty acids is hydrolysed more slowly than corresponding diesters with saturated or monounsaturated fatty acids.

Based on this experiment, the esterification with an omega-3 concentrate gives slower hydrolysis to free astaxanthin, and one should therefore expect lower biological uptake than in feeding experiments with astaxanthin dipalmitate that have been described in the literature.

5

Example 2

A similar experiment as Example 1 was performed with enzymes from salmon
10 intestinal fluid. The intestinal fluid from salmon was collected as described from cod (Lie, Ø. et al., Comp. Biochem. Physiol., 80B (3), (1985), pp. 447-450). The following results were obtained after 45 hours at room temperature:

15	Diester	Free astaxanthin (%)
	18:1	1
	16:0	1
	10:0	6
	EPA+DHA	25

20

Surprisingly, here a considerably higher conversion to free astaxanthin from the EPA+DHA-diester was obtained than from the other diesters. The dielaidate and the dipalmitate were after 45 hours hydrolysed to a degree of just 1%, while the didecanoate was hydrolysed to a degree of 6% and the EPA+DHA diester to a
25 degree of 25%. These results were most unexpectedly opposite of those from Example 1. It is hereby shown that a higher biological uptake than what was achieved in the experiment with astaxanthin diesters that has been described in the literature, probably will be obtained when feeding with an astaxanthin diester prepared with either a concentrate of omega-3 fatty acids or a short chain
30 carboxylic acid.

Example 3

The EPA/DHA astaxanthin diester (ACD) from Example 1 and 2 (30 mg/kg calculated as free (i.e. unesterified) astaxanthin) was added to the pellet by pilot
5 plant production of fish fodder. Urea (2% weight relative to fish oil) was added with water during extrudation. The addition of pigment and fat/oil was done by vacuum-coating of the extruded pellet. The EPA+DHA diester was added together with the fish oil. 200 mg/kg ascorbic acid had been added to the fish oil. After production, a weight average of 18.4 mg/kg was recovered in the pellet (18.1
10 mg/kg as diester, and approx. 0.3 mg/kg as hydrolysed ester). This shows that the EPA+DHA diester survives the production process in sufficient degree to be utilised in practical feeding of fish. Several batches of this fodder composition was produced. Detailed analytical data are given in the calculation below.

15 Commercial astaxanthin (Carophyll Pink, Roche, 30 mg/kg calculated as free astaxanthin) was added to fish fodder in the same manner as above. In this commercial product unesterified astaxanthin is finely dispersed in a starch-covered matrix of gelatine and carbohydrates. Ethoxyquin and ascorbyl palmitate are added as antioxidants. The process and raw materials, including urea addition, were
20 identical with what is given above, with the exception that the fish oil contained no ascorbic acid. After production, a weight average of 25.0 mg/kg free astaxanthin was recovered in the pellet. Several batches of this fodder composition was produced. Detailed analytical data are given in the calculation below.

25 The two fodder compositions were given to salmon. Initially, the average weight of the fish was 70 g. In the calculations that follow, we do not include pigment content of fish before feeding started. The values were identical for both groups, and the numerical values are so small that they will not be significant for the conclusions.

30 After 8 months the average weight of 20 fish for each group was 1180 g for the fish fed with astaxanthin diester, and 1153 g for the fish fed with commercial pigment. The average astaxanthin content of the fish fillet was 3.34 mg/g for the fish fed with the diester and 3.23 mg/g for the fish fed with commercial pigment. If we assume

that the fish weight consisted of 70% muscle, we have the following average values for astaxanthin content:

Fish fed with astaxanthin diester: $1.180\text{kg/fish} \times 0.70 \times 3.34\text{ mg/kg} = 2.76\text{ mg}$

5

Fish fed with commercial astaxanthin: $1.153\text{kg/fish} \times 0.70 \times 3.23\text{mg/kg} = 2.61\text{ mg}$

The fish fed with astaxanthin diester had received the following fodder:

10	457.4 kg containing 18.4 mg/kg	=	8416 mg
	97.6 kg containing 17.1 mg/kg	=	1669 mg
	32.6 kg containing 17.9 mg/kg	=	<u>584 mg</u>
	Sum	=	<u>10669 mg</u>

15 Divided by the average number of fish during the study, each fish had received:

$$10669\text{ mg}/762 = 14.00\text{ mg astaxanthin.}$$

This gives a bioavailability of $100\% \times 2.76\text{mg}/14.00\text{ mg} = \underline{19.7\%}$.

20

The fish fed with commercial astaxanthin had received the following fodder:

	463.2 kg containing 25.0 mg/kg	=	11 580 mg
	101,0 kg containing 24.8 mg/kg	=	2 505 mg
25	32.5 kg containing 30.0 mg/kg	=	<u>976 mg</u>
	Sum	=	<u>15061 mg</u>

Divided by the average number of fish during the study, each fish had received:

$$15601\text{ mg}/775 = 20.13\text{ mg astaxanthin.}$$

30

This gives a bioavailability of $100 \times 2.61/20.13 = \underline{13.0\%}$.

Thus feeding with ACD surprisingly gives enhanced biological absorption compared to free astaxanthin.

Example 4 and 5Isolation of crude enzyme mixtures from salmon and trout intestine

5

Tris buffer (0.25 M) solution was prepared by dissolving Tris (4.54g ; 37.5mmol) in 150 ml of distilled water. The pH of the buffer solution was adjusted to 8.0 by adding 2 M hydrochloric acid. All the hydrolysis was done at room temperature so no special effort was necessary.

10

The astaxanthin diester was weighed accurately (~ 40 mg, 33 mmol) along with 2.5 g of chremophor EL emulsifier (from BASF) into 100 ml Erlenmeyer flask. The solution was stirred vigorously for 20 minutes (until homogeneous) and then 20 ml of the Tris buffer was added and stirred for additional 10 minutes. The solution was suction filtered and the red filtrate was poured into 25 ml volumetric flask and diluted to the mark with buffer. Concentration of all the solution was determined spectrophotometrically (492 nm) to be about 1 mg/ml, except for diacetate which was obtained in a lower concentration. All the solutions were stored in refrigerator wrapped in aluminium paper.

20

Isolation of the crude enzyme mixture

Fresh salmon and rainbow trout, that had been fed in the last 12 hours, were obtained from local fish farms. At the laboratory the fresh fish was cut open and the crude enzyme mixture extracted as follows: From the digestion line there were a lot of narrow tubes surrounded by fat tissue. Each of them was cut off and extracted by hand into a cooled container. The viscous solution (~50 ml) was diluted to 100 ml with 0.25 M Tris buffer and stirred for 30 minutes in an ice bath. Next step was centrifugation at 11.000 rpm for 20 minutes at 4°C (RC5C from Du Pont). The water layer was extracted by pipette to another centrifuge glasses and the centrifugation repeated at 18.000 rpm for 30 minutes at 4°C. The water layer was transferred to Erlenmeyer flask and quickly frozen by liquid nitrogen. Before starting the reaction the mixture was thawed and purified by ultracentrifuge at 27.000 rpm for 45 minutes at 4°C (157.000 g at top). The lipase preparation was obtained as a clear yellow solution.

Hydrolysis

Into a 10 ml round bottom flask was added 2 ml of stock solution and 3 ml of crude enzyme mixture from fish intestine. The flask was filled with nitrogen before
5 closing, wrapped in aluminium paper and stirred for 48 hours. When finished, all the water was removed under reduced pressure (0.01 Torr) and redissolved in CH_2Cl_2 . The solution was filtered through cotton wool plug and stored under nitrogen in a closed container. Analytical TLC was used to monitor the progress of the reaction with 5% acetone in CH_2Cl_2 as eluent (R_f = 0.0-0.1), Monoest. (R_f
10 = 0.3-0.5), Diest. (R_f = 0.8-0.9)). To determine the degree of hydrolysis all the samples were injected into analytical HPLC. [*Eluent*: 30% acetone in n-hexane. *Column*: Nucleosil 50-5 (2x30 cm) column. *Flow rate*: 0.15 ml/min. *Detector*: 470 nm (R_f = 0.4-0.5, Monoest. R_f = 0.7, Diest. R_f = 0.9-1.0)].

15

Example 4

A similar experiment as in Example 2 was performed with enzymes from salmon intestinal fluid.

20

The diesters that were hydrolysed were prepared by esterification of astaxanthin with the following carboxylic acids:

Acetic acid, C16:0 (palmitic acid), fatty acids from a fish oil rich in omega-3 fatty
25 acids (approx. 18% EPA and 12% DHA, called K30 in the tables below), a concentrate of omega-3 fatty acids (approx. 30% EPA and 20% DHA, called K55 in the tables below), the same concentrate of EPA and DHA as used in example 2 (approx. 50% EPA and 35% DHA, called ACD in the tables below), purified EPA (more than 95% EPA, called EPA in the tables) and purified DHA (more than 90%
30 DHA, called DHA in the tables).

Table 1 shows the degree of hydrolysis after 48 hours hydrolysis with crude enzyme mixture from salmon intestine.

After 48 hours the diester of astaxanthin with palmitic acid (16:0) had resulted in no free astaxanthin. However, it is known from the prior art, that this diester is hydrolysed in salmon and gives rise to an increased level of pigment in the fish. Thus, this result indicates that the crude enzyme preparation was not in an optimal condition, presumably due to proteases which may act to degrade the needed enzymes.

All other diesters with omega-3 fatty acids or a short chain carboxylic acid (acetat) gives rise to a higher hydrolysis of diester to diol compared to the diester of astaxanthin with palmitic acid and will therefore be expected to give rise to a higher uptake of pigment in the fish.

The highest degree of hydrolysis was observed with the diester ACD (9%). The relative amount of free astaxanthin was three times as high as that from the diester of a lower concentrated fish oil (K30). The results further show that the diester of purified EPA is hydrolysed faster than the diester of DHA. However, surprisingly the concentrate of EPA and DHA called ACD is hydrolysed faster than purified EPA. The diester of acetic acid is hydrolysed to 7% free astaxanthin. However, it should be noted that for this compound only 13% remains as diester when the reaction is ended. This is much lower then for the other products that were tested, and indicates that if the reaction time had been prolonged, a high amount of free astaxanthin would have been obtained from this diester. Similarly, the only 27% of the diester of EPA remains unreacted. Accordingly, under in vivo conditions, it is expected that the diesters may show even higher relative degree of hydrolysis to free astaxanthin than what is demonstrated in table 1.

Example 5

A similar example as Example 2b was performed. However, for this Example crude enzyme preparation from rainbow trout (*Oncorhynchus mykiss*) was used. The results are shown in Table 2. Table 2 demonstrates that after 48 hours 71% of the diester of palmitic acid remained unreacted. For the fish oil 37% was unreacted, while for ACD only 14% remained unreacted. As for salmon, we observe that EPA

reacts faster than DHA (17 and 21% unreacted), however for rainbow trout we observe that an omega-3 concentrate like the ACD diester gives higher degree of hydrolysis than the diester of purified EPA. Only very small amounts of diester of acetic acid (3%) remains, indicating that a diester of astaxanthin with a short chain
 5 carboxylic acid will give very rapid hydrolysis in fish intestine.

These results are confirmed by looking into the relative amounts of free astaxanthin in table 2. Here ACD shows the highest results, with 27% free astaxanthin, compared to only 11% for the diester of palmitic acid.

10

The results in Tables 1 and 2 below support the conclusions that an astaxanthin diester with a concentrate of omega-3 acids or an astaxanthin diester with short chain carboxylic acids will give higher biological uptake in farmed fish than experiments with astaxanthin diesters that have been described in the literature.

15

Table 1. Ratio (%) between astaxanthin diester, monoester and diol, after 48 hours hydrolysis with crude enzyme mixture from salmon intestine.

Substrate	Diesters (%)	Monoesters (%)	Diol (%)
Acetate	13	80	7
16:0	81	19	0
K30	61	36	3
K55	42	52	6
ACD	37	54	9
EPA	27	67	6
DHA	44	53	3

20

Table 2. Ratio (%) between astaxanthin diester, monoester and diol, after 48 hours hydrolysis with crude enzyme mixture from rainbow trout intestine.

Substrate	Diesters (%)	Monoesters (%)	Diol (%)
Acetate	3	83	14
16:0	71	18	11
K30	37	46	17
K55	25	52	23
ACD	14	59	27
EPA	17	58	25
DHA	21	55	24

25

The concentrate of omega-3 fatty acids comprising approximately 50% eicosapentaenoic acid (EPA) and approximately 35% docosahexaenoic acid (DHA)(in total more than 90% omega-3 fatty acids) which is used in Example 3 to prepare the astaxanthin diester, is only one example and shall not be considered
5 as limiting for the invention. A person skilled in the art will understand that other concentrates of omega-3 fatty acids, e.g. concentrates containing less than 90% omega-3 fatty acids, might be used for preparing a astaxanthin diester giving similar results as those shown in the examples. Astaxanthin diesters esterified with carboxylic acids from marine oils containing omega-3 carboxylic acids or prepared
10 from concentrates of omega-3 fatty acids comprising a total amount of EPA and DHA from 18 to 100 %, preferentially from 40 to 100%, are covered by this invention. More precisely astaxanthin diesters prepared from concentrates of omega-3 fatty acids comprising an amount of EPA from 8 to 98 %, preferentially from 25 to 98%, and an amount of DHA from 8 to 98 %, preferentially from 15 to
15 98%, are covered by this invention.

Example 2 demonstrates that a diester with a short chain carboxylic acid with enzymes from salmon intestines also gives faster hydrolysis to free astaxanthin than a diester with palmitic acid. Thus such a diester will also be suitable as a
20 pigment with high stability and enhanced biological uptake compared to commercial pigment formulations. In Example 2, we used decanoic acid. Other short chain carboxylic acids will be carboxylic acids with a chain length C1-C12. For the person skilled in the art, it is evident that these acids can be both saturated and unsaturated.

25

Furthermore, the person skilled in the art will see that the invented astaxanthin diester might be stabilised in the same way as commercial astaxanthin pigments, i.e. by means of gelatine-matrix. The amount of gelatine-matrix relative to the amount of pigment may be less for the invented pigment than what is needed to
30 stabilise commercial pigments. Alternatively or additionally the invented pigment might be stabilised by antioxidants and/or by urea as described in our Norwegian patent application no. 19983050.

For a person skilled in the art it is obvious that a fatty acid diester of astaxanthin is more fat soluble than free astaxanthin. This is of course an advantage by the present invention because the pigment will be easier to formulate in the fish feed which is rich in fat.

5

Astaxanthin can be produced from microbial sources. Typical examples of promising microbial astaxanthin sources are the yeast *Phalli rhodozyma* and the alga *Haematococcus pluvialis*. *Phalli rhodozyma* has astaxanthin in free form, while *Haematococcus pluvialis* astaxanthin is present mainly (87%) as diesters

- 10 (Johnson, E.A. and An, G-H. 1991. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* 11(4):297.) For the person skilled in the art it is obvious that based on the present invention, increased bioavailability of astaxanthin diester from microbial sources can be obtained by choosing microbial strain, and/or by feeding with suitable fatty acids, or precursors for such fatty acids, or by choosing other suitable
- 15 fermentation conditions, so that the astaxanthin diester that is produced contain omega-3 fatty acids and/or short chain carboxylic acids.

CLAIMS

1. Pigment comprising a diester of astaxanthin prepared with a carboxylic acid, characterised in that the carboxylic acid is an omega-3 fatty acid and/or a
5 carboxylic acid having from 1-12 carbon atoms.
2. Pigment according to claim 1, wherein the omega-3 fatty acid comprises a total amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) and/or docosahexaenoic acid (DHA) (all cis C22:6 n3) from 18 to 100 %.
- 10 3. Pigment according to claim 1, wherein the omega-3 fatty acid comprises a total amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) and/or docosahexaenoic acid (DHA) (all cis C22:6 n3) from 40 to 100 %.
- 15 4. Pigment according to claim 1, wherein the omega-3 fatty acid comprises an amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) from 8 to 98 % and/or an amount of docosahexaenoic acid (DHA) (all cis C22:6 n3) from 8 to 98 %.
5. Pigment according to claim 1, wherein the omega-3 fatty acid comprises an
20 amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) from 25 to 98 % and/or an amount of docosahexaenoic acid (DHA) (all cis C22:6 n3) from 15 to 98 %.
6. Pigment according to claim 1, wherein the omega-3 fatty acid comprises approximately 50 % eicosapentaenoic acid (EPA) (all cis C20:5 n3) and
25 approximately 35 % docosahexaenoic acid (DHA) (all cis C22:6 n3).
7. Feed for salmonides comprising 25-70 % by weight of proteins, 5-60 % by weight of lipids and 0-40 % by weight of carbohydrates, and pigment in combination with 0-15 % by weight of one or more additional components; such as
30 fillers, adhesives, preservatives, vitamins and minerals, wherein the pigment is a diester of astaxanthin prepared with an omega-3 fatty acid and/or a short chain carboxylic acid.

8. Feed according to claim 7, wherein the omega-3 fatty acid comprises a total amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) and/or docosahexaenoic acid (DHA) (all cis C22:6 n3) from 18 to 100 %.

5 9. Feed according to claim 7, wherein the omega-3 fatty acid comprises a total amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) and/or docosahexaenoic acid (DHA) (all cis C22:6 n3) from 40 to 100 %.

10. Feed according to claim 7, wherein the omega-3 fatty acid comprises an
10 amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) from 8 to 98 % and/or an amount of docosahexaenoic acid (DHA) (all cis C22:6 n3) from 8 to 98 %.

11. Feed according to claim 7, wherein the omega-3 fatty acid comprises an
15 amount of eicosapentaenoic acid (EPA) (all cis C20:5 n3) from 25 to 98 % and/or an amount of docosahexaenoic acid (DHA) (all cis C22:6 n3) from 15 to 98 %.

12. Feed according to claim 7, wherein the omega-3 fatty acid comprises approximately 50 % eicosapentaenoic acid (EPA) (all cis C20:5 n3) and approximately 35 % docosahexaenoic acid (DHA) (all cis C22:6 n3).

20

13. Use of a diester of astaxanthin prepared with an omega-3 fatty acid and/or a short chain carboxylic acid for pigmentation of salmonides.

14. Use of a diester of astaxanthin prepared with an omega-3 fatty acid and/or a
25 short chain carboxylic acid as a pigment in feed for salmonides.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 00/00129

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A23K 1/16, A23K 1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI; AQUATIC SCI & FISH ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	File WPI, Derwent accession no.1991-145985, DAINIPPON INK & CHEM KK: "Prepn. of crushed plant material for use as foodstuff, etc.- by culturing e.g. haematococcus producing astaxanthin(s), drying and crushing"; & JP,A,3083557, 19910409, DW199120	1-14
	--	
A	Marine Biology, Volume 122, 1995, Virtue, P. et al, "Biochemical composition of Nyctiphanes australis and its possible use as an aquaculture feed source: lipids, pigments and fluoride content" page 121 - page 128	1-14
	-- -----	

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 July 2000

Date of mailing of the international search report

26-07-2000

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Dagmar Järvman/gh

Telephone No. +46 8 782 25 00